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New generation adenoviral vectors improve gene transfer by coxsackie and adenoviral receptor-independent cell entry

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New generation adenoviral vectors improve gene transfer by car-independent cell entry. Adenoviral (Ad) vectors possess many attributes that have lead to their use as gene delivery agents in human clinical trials. However, gene transfer efficiency has generally been less than that needed for meaningful clinical responses. The restricted tropism of the virus for its native receptor, the coxsackie and adenoviral receptor (CAR), is emerging as a key limitation to the use of these agents. By developing strategies to achieve Ad infection via alternate receptor pathways, enhanced and more specific gene delivery can be achieved. This new generation of tropism-modified agents holds promise for the improved clinical utility of Ad vectors for gene therapy.

Adenoviral (Ad) vectors have been used for a large number of gene therapy clinical trials, chiefly because of their high in vivo gene delivery capabilities in comparison to other vectors [1]. Nevertheless, gene transfer efficiencies have been less than ideal and have compromised efforts to achieve meaningful clinical responses. For example, in human trials for cystic fibrosis, resistance of the target airway epithelial cells to Ad vector infection was observed [2–5]. Furthermore, several cancer gene therapy approaches based on in vivo gene delivery have also achieved suboptimal levels of gene transfer [6–9]. Gene transfer to vascular tissues is also relatively inefficient [10]. These findings indicate that the current generation Ad vectors may not be adequate to achieve meaningful clinical outcomes in many of the proposed, and endeavored, clinical gene therapy trials.

An understanding of the basic biology of Ad infection helps to explain the suboptimal clinical results and also suggests rational strategies for improvement. The adenovirus is an icosahedral particle with 12 fibers projecting outward from the vertices (Fig. 1) [11]. During the assembly phase of viral replication, fiber monomers trimerize in the cytoplasm and then bind to a viral penton base protein that is subsequently incorporated into the viral capsid. At the distal tip of each fiber monomer is a globular region referred to as the knob domain. It is this knob region that binds to the cellular Ad receptor, initially anchoring the virus to the cells. This receptor, the coxsackie/adenoviral receptor (CAR), binds the majority of human serotype Ads and group B coxsackie viruses [12–14]. Following attachment, viral entry requires a second step, involving an interaction between Arg-Gly-Asp (RGD) motifs in the penton base and cell surface integrins (chiefly $\alpha_v\beta_3$ or $\alpha_v\beta_5$), which then leads to endocytosis of the virion [15]. In the endosome, the virus undergoes a stepwise disassembly and endosomal lysis occurs (a process mediated by the penton base and low endosomal pH), followed by transport of the viral DNA to the cell nucleus [11]. This endosomolysis step is critical for efficient gene delivery, and the ability of Ad to effect endosomal escape is one of the key factors in its efficiency as a vector [16].

From the foregoing, it can be seen that a relative deficiency or inaccessibility of either CAR or cellular integrins could potentially limit the capacity of the Ad vector to accomplish efficient gene delivery. Indeed, recent studies of the airway epithelium have noted a virtual absence of CAR on the accessible apical surface of these cells [17]. This observation clearly contributes to the unfavorable outcomes noted in the cystic fibrosis clinical trials. In addition, a relative deficiency of CAR has been noted in the context of a variety of other Ad-resistant targets [18–22]. Analysis of CAR levels in primary ovarian cancer cells from malignant ascites has shown that these cells are variably low in CAR expression and that this correlates to poor transducibility with Ad vectors [23]. Currently, there is little direct information about CAR expression in other primary tumors, but CAR deficiency has been noted in several established and primary cell lines, including melanoma, pancreas, lung, and glioma

Key words: gene transfer, adenoviral infection, coxsackie virus, knob domain.

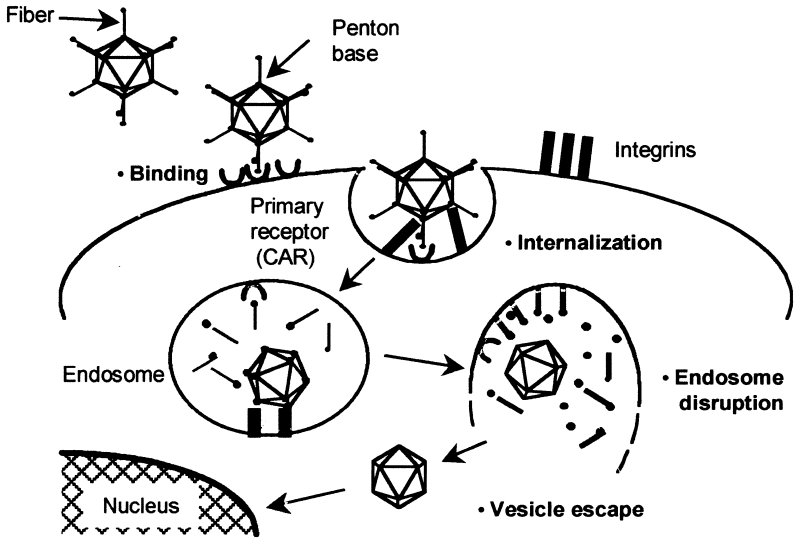

Fig. 1. The pathway of adenoviral infection. Primary binding is mediated by the interaction of the knob domain of the Ad fiber with the cellular receptor, CAR. This is followed by an interaction between RGD motifs in the penton base with cellular integrins, which mediates internalization of the virion into an endosome. Endosomal disruption occurs via mechanisms involving the penton base and the low endosomal pH. The partially disassembled virion particle is then transported to the nuclear membrane for release of viral DNA into the nucleus.

[18, 24, 25]. In these analyses, a direct correlation between CAR expression and infectability with Ad vectors has also been noted. In other studies using primary material, CAR deficiency has been inferred by Ad resistance to infection, which has successfully been overcome by retargeting strategies as discussed later in this article (for example, to head and neck tumor) [20]. Although integrin expression also plays a role in the cell-entry pathway of Ad, for the most part, CAR levels have emerged as the dominant limiting factor as integrins are more widely expressed. Importantly, the key issue of CAR expression has not received significant attention in the design of Ad vector-based gene therapy trials to date. Thus, for the achievement of efficient gene delivery to cells in clinically relevant settings, the exploitation of CAR-independent cell entry pathways may provide a generalized means to circumvent CAR deficiency, which may be broadly relevant to many target cells, including tumors and normal parenchyma. At the very least, it is clear that some understanding of the level of CAR expression on the particular cells one wishes to treat is needed before embarking on protocols using native-tropism vectors. For the treatment of tumors, this suggests the need to include CAR analysis of primary material from individual patients in the pretreatment evaluation, in much the same way as other tumor markers have been used to help guide rational therapeutic decisions.

In an effort to overcome the tropism limitations of Ad, strategies have been devised to redirect the vector to alternative cellular receptors. If one could target to receptors that are expressed on the cell surface at higher levels than CAR, improved efficiency could be achieved. Several strategies to achieve such CAR-independent gene transfer have been devised. In one approach, this goal has been addressed by the development of bispecific targeting adapters, which simultaneously bind to Ad and to a cellular receptor (Fig. 2) [26]. These adapters typically have been designed to have specific recognition for the knob domain of the Ad fiber protein. The rationality of this approach was that one could simultaneously ablate native tropism and impart new tropism, thereby potentially improving both efficiency and specificity of Ad infection. Successful retargeting of Ad has been achieved using a variety of chemical conjugates between an anti-knob Fab and natural ligands specific for cell surface receptors [folate [26], fibroblast growth factor (FGF) [27–30], tetanus toxin fragment [31]), as well as antireceptor antibodies (including anti-epidermal growth factor (anti-EGF-R) [18], anti-EpCAM [32], anti-TAG-72 [23], and anti-CD40 [33]].

Employment of this retargeting approach has established several key concepts for achieving improvements in Ad vector efficiency. First, it is now well established that Ad can achieve effective gene delivery via CAR-independent cellular entry pathways. Thus, the targeted virion's interaction with CAR did not appear to be crucial to its effective cellular entry capacity and the critical subsequent steps of endosomal escape. Second, CAR-independent cell infection allowed enhanced levels of gene transfer improving the susceptibility of many target cells in vitro and, at least locoregionally, in vivo [34]. Third, the new target receptor did not necessarily need intrinsic internalization capabilities, suggesting the native penton-base integrin interaction may still provide this function, thereby broadening the range of potential targets one could consider. Fourth, for those cells that lack both CAR and integrins (for example, T lymphocytes), retargeting could achieve both of these functions (in this case by targeting to CD3 [35]). Fifth, enhanced infectivity was shown to translate to improved therapeu-

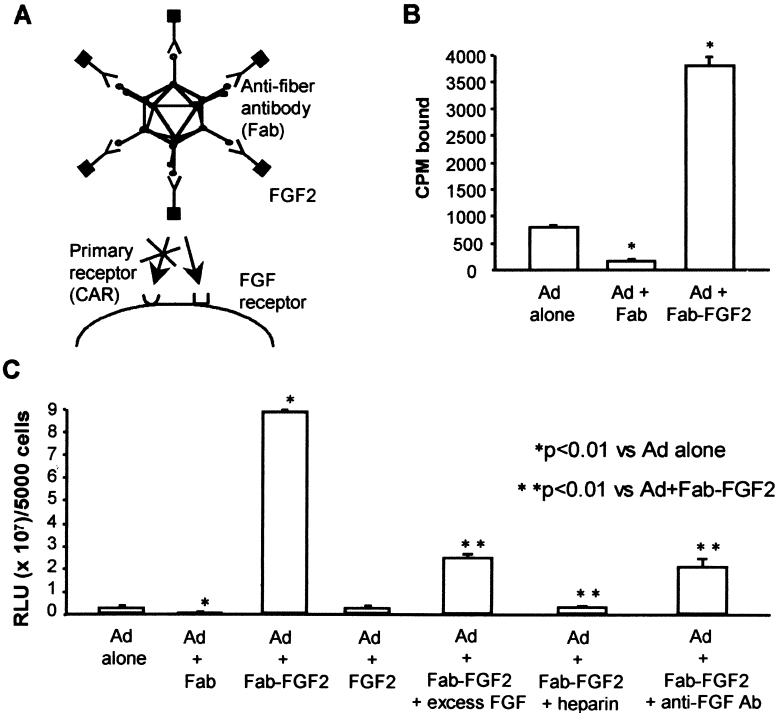

Fig. 2. Redirection of adenoviral infection mediated by conjugate of FGF2 with the Fab fragment of an antiknob-neutralizing mAb achieves enhanced gene delivery. (*A*) Retargeting schema. (*B*) ^{3}H-labeled Ad vector alone or after incubation with Fab or Fab-FGF2 was bound to human umbilical vein endothelial cells at 4°C for one hour. Following washing, cell bound radioactivity (CPM) was measured in a scintillation counter. (*C*) The optimal neutralizing dose of Fab or Fab-FGF2 was incubated with 10^{8} particles of an Ad vector carrying the luciferase reporter gene (AdCMVLuc). After 30 minutes, the complexes were added in triplicate to 24-well plates containing vein endothelial cells. After incubation for 24 hours at 37°C, cells were lysed, and extracts were assayed for luciferase activity. Specificity of FGF2 retargeting was confirmed by blocking with heparin, excess FGF, or anti-FGF antiserum. Free FGF2 alone had no stimulatory effect on Ad-mediated luciferase expression (adapted from Reynolds et al [29]).

tic outcome [36]. Using a locoregional murine model of ovarian carcinoma, two key principles were established: improved outcome at equivalent Ad dose with targeting or similar outcome at a lower dose of Ad particles when targeting was used, the latter result thus suggesting a mechanism to reduce the well-recognized dose-dependent toxicity associated with Ad vectors. Subsequent studies also indicated that retargeting could reduce ectopic hepatic transgene expression and thereby mitigate against the hepatic toxicity associated with suicide gene therapy for cancer [30]. Sixth, the targeting moiety itself may act to improve the overall biological activity of the gene delivery strategy, as with the use of anti-CD40 targeting to enhance dendritic cell activation in response to an Ad-delivered DNA vaccine [37].

Recently, the development of antibody targeting conjugates has been taken a step further with the demonstration that this approach also has a degree of fidelity in the stringent context of in vivo systemic vascular administration [38]. In this study, a conjugate that targets to angiotensin-converting enzyme was used to target Ad selectively to pulmonary vascular endothelial cells upon tail vein injection into rats. This approach achieved a greater than 20-fold enhancement of transgene expression in the target cells, while simultaneously reducing infection of nontarget organs, especially the liver, the predominant site of Ad vector sequestration. Thus, the twin goals of enhancement and selectivity were achieved and may ultimately be applied to gene therapy approaches for pulmonary vascular disease. In the broader context, this study has encouraging implications for the continued development of targeted Ad vectors, although the issue of non-receptor–mediated sequestration of vector by the reticulo–endothelial system still needs to be addressed. Furthermore, this study and those using an FGF conjugate are beginning to address one of the key concerns of the conjugate approach, that being whether the bond between conjugate and virus is sufficiently stable to enable systemic application. In contrast to some earlier predictions, the results so far in this regard are encouraging.

The technical achievement of Ad retargeting via protein complexes has now been approached by a variety of methods. In addition to attachment to the Ad knob, bispecific antibodies have also been derived that bind at the penton base and reduce native tropism for CAR by steric hindrance [39, 40] or attach to Ad hexon protein [41]. Recombinant antibody strategies have also been used, for example, a fusion protein consisting of an anti-knob single chain antibody (scFv), and EGF has been constructed [42]. Dmitriev et al recently developed an alternate approach, using a complex consisting of the ectodomain of CAR in fusion with EGF [43]. This recombinant fusion protein effectively retargeted the vector via non-CAR pathways to EGF-positive cells. Recombinant strategies such as this may indeed offer a more efficient and reproducible means to produce targeting conjugates than chemical linkage approaches.

Advances are also being made in the engineering of ligands per se. In addition to natural ligands (and frag-

ments) and antibodies (including Fab fragments and scFvs derived thereof), phage-panning approaches have been used to identify novel peptide sequences with targeting specificity [44]. These peptides have then been successfully incorporated into targeting conjugates and have achieved targeting fidelity in vitro [45–47]. This combination of target definition with targeted delivery still further broadens the prospects for the development of improved vectors.

The use of conjugate-based targeting approaches has the advantage that a multitude of established targeting moieties can readily be incorporated into the system. Furthermore, many Ad vectors containing different expression cassettes can quickly be combined with any targeting moiety, thereby offering a high degree of flexibility. However, this "two-component" approach adds a degree of complexity to the system, which may not be ideal for ultimate clinical application. Thus, vector tropism modification has also be achieved by genetic methods, in an effort to produce a single targeted vector particle [48]. As Ad capsid proteins are the basis of viral binding and internalization, these proteins are the focus of this approach. Wickham et al have altered the Ad fiber gene, thereby adding cell-binding peptides to the carboxy terminus of the translated protein [49]. Evaluation of viruses possessing these fibers showed that enhanced infection and gene transfer could be achieved by using a limited range of targeting ligands. Using a vector in which an integrin-binding RGD motif had been added to the C terminus of the fiber, enhanced, CAR-independent gene delivery was achieved to human vascular endothelial cells in culture and to the cortical vasculature of the rat kidney by ex vivo infusion of vector into the renal artery. In a separate study, a vector having a C-terminal polylysine addition to enhance binding to heparin sulfates achieved improved gene delivery to the vascular smooth muscle cells of the porcine iliac artery in a balloon injury model [50]. Thus, these studies established the feasibility of enhanced gene delivery through genetic fiber modification.

Although carboxy-terminus modifications of fiber were able to establish an important principle, this location may be suboptimal for ligand addition. The major problem in this regard is that the upper limit of size for incorporated sequences is quite small before the structural integrity of the knob trimer is disrupted. Furthermore, crystal structure analysis of the knob domain indicates that the C terminal is not in an ideally accessible location. More recently, the HI loop of the knob has become a popular site for the insertion of targeting ligands. This choice of this location was pioneered by Krasnykh et al and is based on the crystal structure of the knob domain [51] where the HI loop extends outward and appears accessible for targeting purposes (Fig. 3).

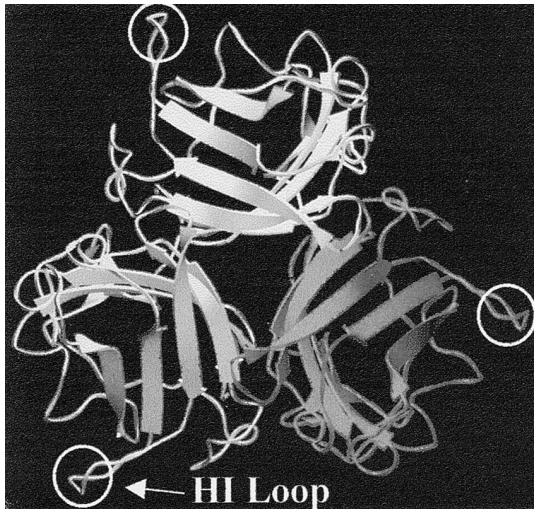

Fig. 3. Genetic modification of the HI loop of the fiber knob domain. The knob trimer, viewed along the three-fold symmetry axis (picture courtesy R. Gerard, adapted from Xia et al [51]). The position of the HI loops, into which the targeting RGD motif was inserted, is shown. The accessibility of this region for binding to cellular targets is seen.

Krasnykh et al first determined that modifications to the HI loop were compatible with viable virus generation and that inserted sequences were accessible for binding, using a FLAG peptide motif as a convenient marker [52]. Then, based on the loop nature of the region being modified and on the recent identification of constrained cyclic peptides that have targeting potential, Dmitriev et al inserted an RGD-4C sequence into the HI loop [19]. This sequence had been shown to have a degree of specificity for tumor vasculature in studies using a novel in vivo phage-panning technique [53]. Studies of recombinant Ad fiber protein containing the mutation clearly confirmed that this motif could bind to integrins when placed at this site. The resulting Ad vector had markedly enhanced ability to infect CAR-negative cells. Most impressively, when this vector was evaluated using primary tumor tissue, significantly enhanced infectivity was again noted compared with the unmodified vector, in some cases by up to three orders of magnitude [54]. In the context of a conditionally replicative Ad designed to achieve tumor cell killing by viral replication and oncolysis, we have recently shown that infectivity enhancement via the RGD-HI loop modification led to improved therapeutic outcome in a murine model [55].

The addition of an RGD motif into the HI loop clearly altered the infection dynamics of the virus. Furthermore, the in vivo systemic administration of vector also resulted in some modification of the biodistribution of transgene expression [56]. Although liver expression still predominated (consistent with the fact that this vector still recog-

nizes CAR), relative increases in other organs, especially the kidney, implied that the RGD motif was available for target cell interaction in this stringent delivery context and thus that the HI loop might prove a useful site for the introduction of more specific targeting moieties. Subsequent studies have established that peptides of up to 63 amino acids in size could be incorporated at the HI loop without deleterious effects with respect to the quaternary structure of the fiber or viral infection dynamics (unpublished data).

Improving Ad infectivity via the modification of capsid proteins besides knob has also been evaluated. In this regard, Vigne et al introduced an RGD motif into the hexon protein [57]. As there are 720 hexons compared with 36 knob domains on the virus, the greater number of targeting ligands with the hexon approach might offer some advantage. However, as these moieties are located close to the virion surface, their interaction with receptors might be compromised by steric hindrance from the fibers. Nevertheless, a significant enhancement of transduction to CAR-negative cells was achieved in vitro using this approach. Shortening of fiber length to improve hexon accessibility could potentially further improve these results. A rigorous, systematic comparison of the different strategies of fiber and hexon modification with respect to the enhancements achieved has not been reported.

The foregoing genetic modification strategies have achieved enhanced infectivity but have not improved specificity, chiefly because all of the modified vectors retained recognition for CAR. Thus, although they represent a significant advance for many disease applications where delivery can be limited locoregionally, greater specificity is required for broader application. Recently, the genetic manipulation of the Ad5 knob to ablate its natural recognition for CAR and yet still retain the structural integrity of the virus has been achieved [58]. Furthermore, a mutation of the penton base to ablate the natural recognition for integrins has also been accomplished. Nevertheless, the ablation of CAR recognition alone does not significantly reduce vector localization to the liver after systemic administration, nor does it reduce hepatocyte transduction [59, 60]. Preliminary studies using vectors in which the penton RGD has also been ablated still show that the vast majority of vector localizes to the liver (unpublished observations). However, these studies have been performed using vectors that do not have a specific alternate ligand. Interestingly, there is now some in vitro evidence that Ad vectors can also achieve primary attachment to cells by binding to heparan sulfates, although this mechanism is yet to be fully characterized (**Note added in proof**). The relevance of this mechanism in vivo has not yet been reported, however. The superimposition of a specific targeting moiety onto a background of a native tropism-ablated vector is eagerly awaited. Whether such a construct will be possible using the peptide sequences compatible with C-terminal or HI-loop insertion is yet to be determined. The specificity of promising bicyclic ligands defined by in vivo phage panning is exquisitely dependent on correct formation of the cyclic disulfide bonds within the peptide, and whether this can be achieved in the HI loop is uncertain. In fact, thus far, few successful peptide insertions in the HI loop have been reported. Alternate strategies to replace entirely the Ad knob domain with novel proteins that can achieve the correct trimerization of fiber as well as posses specific targeting attributes are now also being developed [61–63]. Such approaches may have much greater flexibility with respect to the potential choice of targeting ligand.

In addition to achieving targeted gene expression via tropism modification, expression can be controlled at the level of transcription, through the use of cell-specific promoters. This strategy can help reduce the level of undesirable transgene expression seen in organs such as the liver and spleen, which have a high propensity for nonspecific vector uptake. However, the use of putative cell-specific promoters in Ad vectors can be difficult because there is often a tendency for the specificity of the promoter to be undermined when placed in the Ad genome because of ill-defined *cis*- or *trans*-acting factors. Nevertheless, several promoters that do retain specificity in this setting are emerging [64–66], and strategies to place insulator sequences before or either side of the expression cassette also help [67]. We recently investigated whether a combination of appropriate transductional and transcriptional targeting approaches would significantly improve the transgene expression profile in vivo compared with either strategy used alone [68]. Thus, we combined the conjugate-based pulmonary vascular targeting approach to angiotensin-converting enzyme, with the use of an endothelial-specific promoter, the promoter for the vascular endothelial growth factor receptor type 1 (f/t-1) [64]. The combination resulted in a remarkable synergistic improvement in the specificity of transgene expression for the pulmonary target site (over 300,000-fold improvement compared with untargeted vector) (Fig. 4). These results represent the first successful combined targeting approach. As technology improves in both target definition and promoter development, many other rational combinations for other tissues and disease could be envisaged.

Aside from issues of receptor-specific transduction and of CAR, a major issue concerning the use of Ad vectors for systemic administration is sequestration of the vector by Kupffer cells. It has been shown that at low vector doses, 90% or more of the injected dose is taken up by the Kupffer cells by phagocytosis and degraded, without leading to transgene expression [69]. This clearly has implications for the issue of Ad-related direct inflammatory responses. Vector-associated toxic-

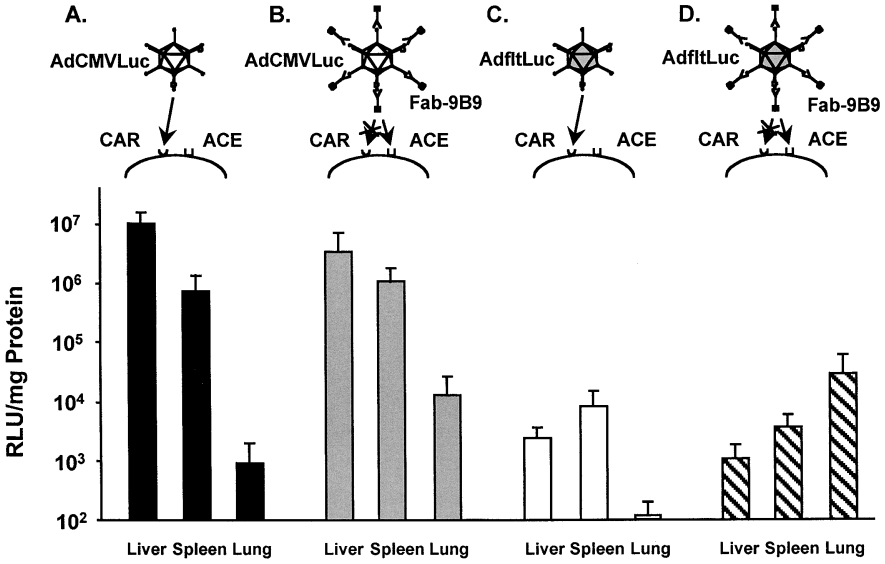

Fig. 4. Combined transductional and transcriptional targeting improves the specificity of transgene expression in vivo. Stepwise improvement in transgene expression in pulmonary endothelium is shown. Rats were injected via the tail vein with vectors carrying the luciferase reporter gene. Then organs were harvested, and luciferase activity was determined three days later. (*A*) Untargeted vector, AdCMVLuc, showing dominant transgene expression in liver and spleen. (*B*) Addition of Fab-9B9 for targeting to angiotensin-converting enzyme leads to enhanced pulmonary gene expression and reduction in liver expression (note log scale). (*C*) AdfltLuc, containing an endothelial specific promoter results in dramatic reduction in expression in all organs. (*D*) AdfltLuc + Fab-9B9 restores transgene expression levels in lungs but still further reduces expression in nontarget liver and spleen, thereby dramatically improving selectivity ratio (adapted from Reynolds et al [68]).

ity observed in human clinical trials is closely linked to the magnitude of particle dose. If Kupffer cell uptake could be avoided, a much lower dose of vector could be administered. Currently, several groups are working on strategies to coat the virus with polymers such as polyethylene glycol. This approach may not only reduce Kupffer cell sequestration but also reduce inactivation of Ad by antibodies [70–72].

In summary, the recent development of targeted Ad vectors has seen significant improvements, but there are still many hurdles to be overcome. Enhanced infectivity has been achieved through both conjugate-based approaches and genetic modification, and such vectors are poised to enter clinical trials in the near future for locoregional disease. For more specific, systemic targeting some form of vector system that incorporates transductional and transcriptional control with strategies to avoid Kupffer sequestration will ensure optimal efficacy. As these systems are developed, the use of the relevant human primary material should be incorporated into the preclinical evaluation as much as possible.

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NOTE ADDED IN PROOF

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